A JOINT STUDY OF MICRODETERMINATION OF URINARY MERCURY—by investigators from three different institutes, in order to establish a standard method

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尿 中 水 銀 の 定 量 法

三研究機関の三人（各一人ずつ）の研究者によって尿中水銀の定量に関する検討を行なった。尿中水銀の正常範囲について議論する場合、その基本となる測定法や測定技術に相違があったのでは同じ水準で評価することはできない。そこで標準となるべき一定の物指しを作る必要がある。著者らは予備実験と本研究の最初の 2, 3 の実験によって、藤村、上田がその酸化処理法を改善した Natelson の方法について検討を加えた。その結果、酸化法を一定にすれば Natelson 法により迅速かつ正確に一致した値をうることができた。また、ポリエチレン試薬瓶に標準水銀液を長時間保存すると、水銀がその表面に吸着されて濃度が低下するので、標準液は調製後直ちに使用すること、また、ポリエチレン瓶は水銀液の保存に不適であることがわかった。

今回の共同実験によって著者らが痛感したことは水銀のみならず、他の産業毒物の測定方法を検査し、その最も良い標準法を紹介するような機関が設立されることであった。そして同じ物指しによって測った結果について検討し合うべきであると考ええる。

Purpose of the experiment

When discussions are made by different investigators on the normal range of a chemical in biological materials, it is meaningless to argue, if there is no standard or agreement of method of analyzing the chemical among the investigators. Although this is quite true and evident, it is not easy to compare the data issued from different investigators on the same basis of evaluations. Recently some papers on the subject of industrial mercury poisoning have appeared and, as mentioned above, a great discrepancy, particularly of normal excretion of mercury in urine was noted. Under such circumstance three authors, each of whom belongs to three institutes, have long been conducting investigation of mercury poisoning. These three authors agreed to make a series of experiments concerning analysis of mercury in urine, in order to establish a standard method among them.

In regard to the microdetermination of urinary mercury innumerable papers have been published. The present experiments were not concerned with those overall methods presented by many previous authors, but a few methods, which had been routinely adopted by the present authors, were examined.

Two of the present authors had been satisfied to use the dithizone-chromatographic method reported by Ashizawa¹⁾. According to this method the digestion of urine is made under a temperature of 60°C, adding sulfuric acid and potassium permanganate. After the digestion, the mercury in the material is combined with dithizone in carbon tetrachloride and is developed through the column of alumina gel, so that the excess dithizone and other disturbing metals, especially copper, are separated from the dithizone mercury, which is then eluted by chloroform ready for colorimetry.

There had been an agreement among the present authors that this method would lead to satisfactory results when fresh urine samples were used. If the urine is rather old, the digestion is not enough to release mercury for combining with dithizone.

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The second method subjected to the present experiment was that introduced by Natelson²⁾. At a laboratory of one of the authors, his colleague, Fujimura³⁾, had closely examined this method. According to his experiment the method is practical, easy to conduct and digestion is sufficient when some modification is made in the oxidation procedure to recover all the mercury, even from the stayed urine. Thus this method was the main one examined for the present study.

Although there was much discrepancy in the results of the initial part of study, coincidental data were finally obtained by finding out why the different results had been yielded.

Planning and Results

The first experiment was so planned that samples of the same urine were distributed to each of the three authors, and the mercury concentration was to be determined by each one's routine technique. There were no other particular conditions arranged among the authors before the experiment. Four samples were prepared by a colleague of one of the authors. The author from Institute "A" used the Natelson's method. The other two authors, of Institute "B" and "C", conducted their assessments by the Ashizawa's dithizone-chromatography method.

Table 1 shows the results, which are quite different in figures of Hg concentration in the same samples. But generally speaking, the values by "B" and "C" are less than those by "A". A large difference is seen in sample

No. 2, because the author from "C" obtained a value of 150 $\mu\text{g/l}$, for which the experiment was made two weeks after the sample had been delivered. Accordingly, most probably, the less recovery of mercury in this case was due to the insufficient oxidation, because of the stayed urine. After long discussion by the authors, a conclusion was reached that the Natelson's method would be preferable for the cooperative experiment.

The second experiment was then conducted by the Natelson's method, modified by Fujimura and Ueda. In this case the three authors discussed what special caution should be taken when using this technique. Table 2 shows the data which reveal recovery tests. HgNO_3 solution of concentration of 100 $\mu\text{gHg/dl}$ was added to three urine samples, with 260 μg , 400 μg and 800 μg respectively. Figures in the brackets of the table are given for the recovered amounts of mercury from each sample. Not only are there great differences between the added amounts and the recovered, but any definite inclination of the difference is not observed. Moreover, significant discrepancies of the results are recognized among the three authors. The results suggested that a third experiment had to be made in order to confirm the influence of the digestion procedure.

In Table 3 the data are described in regard to the influence of oxidation procedure. The experiment in connection with both Table 3 and Table 4 were carried out at a laboratory of Institute "A", with all three authors co-

Table 1 Urinary mercury concentrations of the same samples, estimated by routine method of each of the authors (First experiment).

Urine sample No.	Analytical method	Mercury concentration $\mu\text{g/l}$				Difference between the highest and the lowest value		
		Natelson's method		Dithizone-chromatographic method				
		Institute A	Institute B	Institute C				
1		760	680	700	—	80		
2		800	850	648	656	640 150	420	700
3		350	224 216	156 218	210	260	194	
4		360	240 290	264 304	380	430	140	

Remarks: Amount of urine used for each determination, A: 50 ml, B and C: 20 ml.

Table 2 Second Experiment (Natelson's method)

Urine sample No.	Hg ($\mu\text{g/l}$) added	Mercury concentrations in urine $\mu\text{g/l}$						Difference between the highest and the lowest value ($\mu\text{g/l}$)
		Institute A				Institute B	Institute C	
		1st	2nd	3rd	4th			
1	0	144	168	160	160	120	156	48
2	260	448 (304)	464 (296)	464 (304)	450 (290)	336 (216)	—	128
3	400	—	560 (392)	588 (428)	400 (240)	536 (416)	328 (172)	260
4	800	1320 (1176)	1360 (1192)	1360 (1200)	800 (640)	1604 (1484)	811 (660)	804

Remarks: (1) HgNO_3 solution with concentration of 100 $\mu\text{g/dl}$ was added to the urine of No. 2, No. 3 and No. 4, with 260, 400 and 800 $\mu\text{g/l}$ respectively.

(2) In the brackets, recovered amounts of Hg are shown.

(3) Urine amount for each determination was 50 ml.

Table 3 Same digestion procedure with different extraction processes. (Low temperature digestion by the Ashizawa's method).

Urine sample No.	Urinary Hg conc. ($\mu\text{g}/20\text{ ml}$)	
	Ext. process	
	Natelson	Ashizawa
1	8.6	8.6
2	15.2	16.4
3	10.5	11.9
4	6.4	7.7
5	20.4	24.2

Table 4 Different digestion procedures with same extraction process (the Natelson's)

Urine	Urine sample No.	Urinary Hg conc. ($\mu\text{g}/50\text{ ml}$)	
		Digestion method	
		Natelson	Ashizawa
From workers exposed to inorganic mercury	1	2.2	2.0
	2	3.0	3.5
	3	5.5	5.0
From workers exposed to organic mercury	1	5.4	1.0
	2	6.2	1.5
	3	14.2	2.8
	4	16.2	5.5

operating. When the digestion process was conducted by one method—in the experiment of Table 3 the Ashizawa's low temperature

oxidation was employed—and different extraction procedures (Natelson and Ashizawa) were introduced, the values did not fluctuate very much as seen in Table 3. On the contrary, when different digestion processes (Natelson and Ashizawa) with same extraction procedure (Ashizawa) were tried, much lower values for the urine samples from those exposed to organic mercury were obtained in the oxidation by the Ashizawa's low temperature procedure, in comparison with the modified oxidation of Natelson. The urine samples for the experiments of Table 3 and Table 4 were brought to analysis within 24 hours after collection. In other words the data from Table 3 and Table 4 imply that the extraction process of mercury would not be the cause of the varied results, but the oxidation procedure would be the main cause of unsatisfactory results. Particularly, when urine becomes old or contains organic mercury, less mercury is released to be combined with dithizone, as a result of insufficient digestion. This confirmed that the Natelson's method (modified by Fujimura and Ueda) should be employed at least for the urine digestion. Also, since the extraction technique is much easier in the Natelson's than in the Ashizawa's column chromatography, the authors agreed to adopt the former for the following experiments. Before the authors proceeded to the next experiment, they realized an important

point, i.e., checking the standard Hg solution prepared by each of them. Fortunately all the three had kept the standard solutions of $10 \mu\text{g Hg/ml}$, prepared about one year before the present study started. Also, all of them kept their solutions in polyethylene plastic bottles, which they thought best to keep Hg stable. Nevertheless, this intuitive prediction was not proven, as seen in the results of Table 5. According to their protocols, about one year before the present study the extinction coefficient of the solution was 0.7 for the authors from Institutes "A" and "C", and it was 0.45 for the author from "B", by their own spectrophotometers. However, Table 5 shows much lower figures of extinction coefficient, which were calibrated one year after the preparation. This fact is enough to postulate that some mercury ion would be absorbed into the surface of the plastic, if

Table 5 Examination of old* Hg standard solution and of the reagent, dithizone.

Dithizone of each author		Hg standard solution of each author		
		Extinction coefficient (490 m μ)		
		A	B	C
Dithizone of each author as blank	A	0.235	0.120	0.470
	B	0.220	0.128	0.490
	C	0.270	0.118	0.448
CCl ₄ as blank	A	0.225	0.141	0.485
	B	0.249	0.140	0.512
	C	0.274	0.128	0.461

* The standard Hg solutions were prepared one year before the present study, and kept in bottles of polyethylene plastic.

not all. The data shown in Table 5 mean that the dithizone used by each author is good enough as a reagent.

Then next experiment was to prepare a new standard Hg solution by each of the authors. As shown in Table 6, results quite coincidental with those of the previous protocols were obtained.

Table 6 Examination of new* Hg standard solution.

Hg standard solution of each author	Extinction coefficient E (490 m μ)	
	A	B
**Author		
A	0.69	0.70
B	0.446	0.454
C	0.67	0.69

* The solutions were prepared just before calibration.

** The calibration was made by each author's spectrophotometer.

Before the final experiment was conducted, the three authors tried to make analysis of mercury in urine at the same laboratory by well arranged technique and experience acquired from the above experiments. The results are indicated in Table 7 which gives coincidental figures of data. The recovery test, which was carried out by two authors, resulted also in satisfactory records.

Thus all the factors which had influence on the results of determination became overt. The final experiment was conducted at each author's laboratory by distributing samples of the same urine from a worker exposed to organic mercury. Table 8 shows the close

Table 7 Determination of urinary mercury from same samples at a laboratory in "A" by three authors.

No. of sample	Hg (μg), added to 50 ml of urine	Urinary mercury concentration $\mu\text{g}/50 \text{ ml}$				Difference between the highest and the lowest value
		Author A 1st	Author A 2nd	Author B	Author C	
1	0	5.00 (100)	5.21 (104)	4.90 (98)	5.28 (105.6)	0.38 (7.6)
2	5	10.00	11.68	10.43	—	—
Recovery		5.00	6.47	5.5		

Remarks: () = $\mu\text{g}/1$

Table 8 Urinary mercury concentration of same sample by the three authors in each laboratory. (Natelson's method)

Author	Urinary Hg concentration	
	Hg $\mu\text{g}/50\text{ ml}$	Hg $\mu\text{g}/1$
A	9.87	197.4
B	10.20	204.0
C	10.40	208.0

agreement of results by the three authors.

The conclusion was drawn that the Natelson's method, of which the digestion procedure had been improved by Fujimura and Ueda, should be adopted for the routine determination of urinary mercury.

Comment

There have been reported various methods of mercury determination of biological materials. As already mentioned above, Fujimura had examined several methods and recommended the Natelson's method, which he had modified in the process of oxidation, because the digestion by the Natelson's is not sufficient for the material containing organic mercury, either for old or stayed urine. Main points he modified are as follows: (1) To promote oxidation, he added H_2O_2 which reduced the required amount of KMnO_4 . (2) To complete the oxidation, samples were heated up to

100°C for two hours. In this case a reflux condensor was connected with the digestion flask to collect mercury, vapourized by heating.

In Table 9, a comparison of oxidation procedure and of assessment method by several authors was made, and a general idea of the difference of analytical methods may be reviewed in the table. The Ashizawa's method by column chromatography had been used by preference of two of the present authors, but in this method the oxidation is made under low temperature (up to 60°C) which is not sufficient for organic mercury, as already mentioned. Moreover the technique of elution of dithizone-Hg from the column requires some period of time for training. Miura⁴⁾ developed a method which brought fairly stable results. But, a reagent, di- β -naphthylthiocarbazone, used in that method, is not easily available on the market. One color method introduced by Jacobs and Singerman⁵⁾ was found to give a satisfactory result. However, since the oxidation is made again under low temperature, the same trouble as seen in the Ashizawa's method will be induced. A method reported by Cholak and Hubbard⁶⁾ seemed us to be excellent, but it was considered that his method would not be very practical in case of handling many samples. The A.O.A.C. method⁷⁾ should give a satisfactory result, but the oxidation procedure by

Table 9 Comparison of various analytical methods of mercury determination from urine sample.

Name of author	No. of reference	Urine amount (ml)	Oxidation procedure	Calibration
Stock, A., Lux, H., Cucuel, F., and Köhle, H.	(8)	100	HCl (25 ml) + KClO_3 (2 g), Heating (100°C) with reflux condensor	Electric decomposition Copper electrode, Heating and Cooling, Calibration of diameter of Hg ball
Natelson, S. (Modified by Fujimura and Ueda)	(2) (3)	50	30% H_2O_2 (15~20 ml) + H_2SO_4 (7 ml) + KMnO_4 (2 g), Heating (100°C) with reflux condensor	Eliminate the excess dithizone by 9N- NH_4OH One color of dithizone Hg
A. O. A. C. (Analysis of the Association of Official Agricultural Chemists)	(7)	50~200	HNO_3 (60 ml) + H_2SO_4 (7 ml) Heating (100°C) with reflux condensor	Colorimetry of dithizone-Hg
Ashizawa	(1)	20~50	H_2SO_4 (2~3 ml) + KMnO_4 (5~10 ml) + H_2O (100~150 ml) Heating (60°C) without reflux condensor	Chromatography Colorimetry of dithizone-Hg
Simplified method by Nishimura and Aoki	(9)	50	NaOH (5 ml) + KMnO_4 (20~30 ml) + H_2SO_4 (10~20 ml), Cold digestion	Eliminate the excess dithizone by 9N- NH_4OH Colorimetry of dithizone-Hg

this method is not necessarily required even in case of stayed urine.

In reviewing several methods the Natelson's method was adopted for the present study, and by modifying the oxidation procedure the final experiment resulted in success. Our purpose, of obtaining a standard scale, was realized. One of our harvests from the experiments was to find that Hg ion might be absorbed in the surface of polyethylene bottles.

Conclusion

Three authors from different institutes made cooperative experiments to obtain a standard method for the determination of urinary mercury. Some preliminary experiments and the initial experiments in the present study suggested that the authors use the Natelson's method which had been modified in the procedure of digestion by Fujimura and Ueda, members of the institute of one of the authors. After a series of experiments a satisfactory result was obtained by each of the authors. The key points which one must be careful of when conducting the determination by the Natelson's method was as follows:

(1) The oxidation of urine should be sufficient to make Hg combine with dithizone for the stayed urine or, containing organic mercury.

(2) Potassium permanganate to be added should be minimal. Addition of H_2O_2 is helpful for this purpose.

(3) Standard Hg solution should not be

kept in a bottle made of polyethylene, and should be used as soon as possible after preparation.

(4) Through our experience in this study, it is hoped that an organization could be established in order to examine various methods already published, and to recommend a standard method for the determination of industrial chemicals which may be hazardous to workers' health.

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(受付: 1963年4月13日)